GGL - PRIORITY

C24

The Patent Office

PCT/EP 99/07303

REC'D 11 NOV 1999

WIPO PCT

INVESTOR IN PEOPLE

4

EP99/7303

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed P. Mahoney

Dated 16 September 1999.

Patents Form 1/77

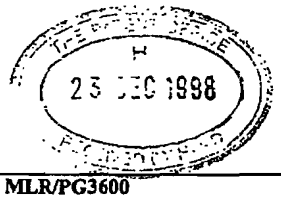
Patents Act 1977
(Rule 16)

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)



The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your Reference — MLR/PG3600

2. Patent ap *(The Paten)* — **9828628.9** — 23 DEC 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

GLAXO GROUP LIMITED
GLAXO WELLCOME HOUSE
BERKELEY AVENUE
GREENFORD
MIDDLESEX
UB6 ONN
GB

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its corporation — GB 473587003

4. Title of the invention — NOVEL LIGAND

5. Name of your agent *(if you know one)* — MARION L REES (SEE CONTINUATION SHEET)

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

GLAXO WELLCOME PLC
GLAXO WELLCOME HOUSE, BERKELEY AVENUE
GREENFORD, MIDDLESEX
UB6 ONN, GB

Patents ADP number *(if you know it)* 6990537001

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of Filing (day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing (day / month / year)

8. Is a statement of inventorship and of right to grant a patent required in support of this request? *(Answer yes if:*

a) any applicant named in part 3 is not an inventor, or

b) there is an inventor who is not named as an applicant, or

c) any named applicant is a corporate body.

YES

See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	1
Description	14
Claim(s)	2
Abstract	-
Drawing(s)	12

10. If you are also filing any of the following, state how many against each item

Priority Documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patent Form 9/77)*	
Request for substantive examination *(Patent Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application

Signature MARION REES 23 December 1998

AGENT FOR THE APPLICANTS

12. Name and daytime telephone number of person to contact in the United Kingdom

CATE WEST

0181-966 8685

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication of communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the patent Act 1977 stops you from applying for a patent abroad without first getting written permission form the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been received

a) Notes

If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form

If you have answered "Yes" Patents Form 7/77 will need to be filed.

d) Once you have filled in the form you must remember to sign and date it.

e) For details of the fee and ways to pay please contact the Patent Office.

Additional Agents
(See Page 1 No. 5)

NAME(S)

Alan HESKETH
William Michael DADSON
Michael ATKINSON
Karen CRAWLEY
Peter I. DOLTON
Hugh B. DAWSON
Wendy Anne FILLER
Ruth Elizabeth HACKETT
Catriona MacLeod HAMMER
Audrey HAMMETT
Graham M.H. LANE
Stephanie Anne LEAROYD
Helen Kaye QUILLIN
Michael A REED
Marion REES
Michael John STOTT
Andrew J. TEUTEN
Rachel M. THORNLEY
Janis Florence VOLCKMAN

ADDRESS

Glaxo Wellcome plc
Glaxo Wellcome House
Berkeley Avenue
Greenford
Middlesex
UB6 ONN
Great Britain

Novel Ligand

The present invention relates to a novel protein of the TNF ligand superfamily, nucleotides coding for it, vectors and host cells containing the same and methods of screening for modulators of the interaction between said protein and its receptor, said modulators for use in therapy for various disorders including, but not restricted to, cancer, inflammation, infection and autoimmune disease. Also, direct use of said ligand in therapy, for example against viral diseases or as a potential vaccine adjuvant.

Fifteen other members of the TNF ligand family have currently been cloned and published and most have been shown to bind to cell-surface receptors of the TNF receptor family. The interaction between a TNF ligand and its receptor is the key signal to start a chain of events leading to a range of responses as diverse as T-cell proliferation, apoptosis and induction of cytokine production. Some activities such as induction of T-cell proliferation are common to many members of the family, whilst some are shared by only a few, and others are unique. The interaction between these ligands and their receptors provides an attractive target for the development of novel therapies.

The present invention provides an isolated protein comprising i) a polypeptide having the amino acid sequence of figure 1 or ii) a variant of the polypeptide of i). The invention also provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 2 or variants thereof. The protein having the amino acid sequence provided in Figure 2 is obtainable from humans and is a type II membrane protein with a single transmembrane domain near the N-terminus, which contains two potential N-linked glycosylation sites, and a protease cleavage site between amino acids glycine121 and glutamate122. The polypeptide having the amino acid sequence provided in figure 1 is soluble, and forms part of the extracellular region of the polypeptide having the amino acid sequence provided in figure 2. Preferably the protein of the invention comprises a polypeptide which is 65%, preferably 75%, more preferably 80% and even more preferably 90% homologous to the amino acid sequence of Fig 1. The protein of the invention most preferably comprises a polypeptide which is, at least 95%, for example 97%, 98% or 99% homologous

to the amino acid sequence of Fig 1.Preferably the protein of the invention is obtainable from mammals, more preferably from mice or humans, and most preferably from humans.

The present invention further provides a protein comprising a polypeptide which has the sequence as provided in figure 2 from amino acid 122 onwards, or the sequence as provided in figure 6 from amino acid 115 onwards.

The present invention further provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 5, or variants therof. Moreover, the invention provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 6 or variants thereof. The protein having the amino acid sequence as provided in figure 6 is isolatable from mice and is a type II membrane protein with a single transmembrane domain near the N-terminus, which protein contains one potential N-linked glycosylation site, and a protease cleavage site between amino acids arginine114 and proline115. The polypeptide having the amino acid sequence provided in figure 5 is soluble, and forms part of the extracellular region of the polypeptide having the amino acid sequence provided in figure 6.

Proteins of the invention isolatable from humans, and proteins of the invention isolatable from mice are highly homologous, displaying 67% amino acid identity over their entire sequence. In the C-terminal region involved in receptor binding, amino acid identity is much higher (87%). A significant difference between proteins of the invention isolatable from humans or mice is the presence of an additional exon in the mouse sequence encoding an extra 31 amino acids which reduces the overall homology between the two proteins.

The term variant refers to proteins which have substantially the same biological functionality as the protein for which sequence information has been provided. The term variant encompasses fragments, derivatives and analogues of the protein of the invention.

Fragments include portions of the protein which retain sufficient identity to the original protein to be effective for example in a screen.

Derivatives include alternate forms of the protein sequence which may have deletions, additions or substitutions of one or more amino acids. It will be understood by a person skilled in the art that certain substitutions, deletions or additions of amino acids can be made, or indeed can occur naturally without substantially altering the function of the protein.

Analogues include but are not limited to precursor proteins which can be activated by cleavage of the precursor protein to produce an active mature protein, or a fusion with a leader or secretory sequence to aid purification.

The protein of the present invention may be a recombinant protein, a natural protein or a synthetic protein.

The proteins of the invention may be present in all embodiments in trimeric form and such trimers form an embodiment of the invention. Typically the protein of the invention will bind to their receptor as a trimer, thus allowing two or more receptor molecules to be brought into proximity.

The present invention also provides antibodies specific for the protein of the invention. The term antibody as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic determinants of proteins of the present invention. The antibodies of the present invention may be polyclonal or monoclonal, may be intact antibody molecules or fragments containing the active binding region of the antibody, e.g. Fab or $F(ab)_2$. The present invention also includes chimeric, single chain and humanised antibodies and fusions with non-immunoglobulin molecules. Various procedures known in the art may be used for the production of such antibodies and fragments.

The proteins of the invention, their variants or cells expressing them can be used as an immunogen to produce antibodies thereto. Antibodies generated against the proteins of the invention can be obtained by direct injection of the polypeptide into an animal, preferably a non-human. The antibody so obtained will then bind the protein itself. In this manner, even a fragment of the protein of

the invention can be used to generate antibodies binding the whole native protein.

The antibodies of the present invention may be used to locate the protein of the invention in tissue expressing that protein. They are also, for example, useful for purification of a protein of the invention, and accordingly there is provided a method of purifying a protein of the invention which method comprises the use of an antibody of the present invention. The antibodies of the present invention may also be used as therapeutic agents in their own right.

A further aspect of the invention provides an isolated polynucleotide which encodes a protein of the invention. Also included within the invention are anti-sense nucleotides or complementary strands. Preferably the nucleotide encodes a protein of the invention isolatable from a mouse or a human. More preferably the isolated polynucleotide comprises the polynucleotide portion having the nucleotide sequence shown in figure 3, which codes for the polypeptide shown in Figure 1, a variant of said portion, or a complementary strand. The present invention further provides an isolated polynucleotide comprising the nucleotide sequence shown in figure 4, which codes for the polypeptide of Figure 2.

The nucleotide sequence may be isolated from a cell (preferably a human cell), by screening with a probe derived from the protein of the invention, or by other methodologies known in the art such as polymerase chain reaction (PCR) for example on genomic DNA with appropriate oligonucleotide primers derived from or designed based on the protein of the invention. A bacterial artificial chromosome library can be generated using mouse or human DNA for the purposes of screening.

The nucleotide sequences of the present invention may be in the form of RNA or in the form of DNA, for example cDNA, genomic DNA, and synthetic DNA. Preferably the nucleotide sequence of the inention is cDNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the protein of the invention may be identical to one of the coding sequences set forth in the Figures, or may be a different coding sequence which as a result of

the redundancy or degeneracy of the genetic code, encodes the same protein as the sequences set forth therein.

A nucleotide sequence which encodes a protein of the present invention may include: a coding sequence for the protein or any variant thereof; a coding sequence for the protein or any variant thereof and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; a coding sequence for the protein or any variant thereof (and optionally additional coding sequence) and non-coding sequences, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the full length protein.

The invention also provides nucleotide variants, analogues, derivatives and fragments which encode a protein of the invention. Nucleotides are included which preferably have at least 65% identity over their entire length to the nucleotide having the sequence of Figure 3. More preferred are those sequences which have at least 75% identity over their entire length to the nucleotide having the sequence of Figure 3. Even more preferred are polynecleotides which demonstrate at least 90%, for example 95%, 97%, 98% or 99% identity over their entire length to the nucleotide having the sequence of Figure 3.

The nucleotide sequences of the invention may also have the coding sequence fused in frame to one or more marker sequences which allow for purification of the protein of the present invention such as a FLAG epitope, a myc sequence, or a secretory signal.

The nucleotide sequences of the present invention may be employed for producing a protein of the invention by recombinant techniques. Thus, for example the nucleotide sequence may be included in any one of a variety of expression vehicles or cloning vehicles, in particular vectors or plasmids for expressing a protein. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences. Examples of suitable vectors include derivatives of bacterial plasmids; phage DNA; yeast plasmids; vectors derived from

combinations of plasmids and phage DNA and viral DNA. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

More particularly, the present invention also provides a vector comprising one or more of the nucleotide sequences as described above. The vectors are, for example, an expression vector, such as a plasmid or viral vector into which an isolated polynucleotide of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the vector further comprises one or more regulatory sequences to direct mRNA synthesis, including, for example, a promoter, operably linked to the sequence. Suitable promoters include: CMV, LTR or SV40 promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The vector may contain an enhancer and a ribosome binding site for translation initiation and a transcription terminator.

Large numbers of suitable vectors and promoters/enhancers, will be known to those of skill in the art, but any plasmid or vector, promoter/enhancer may be used as long as it is replicable and functional in the host.

Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts include mammalian expression vectors, insect expression vectors, yeast expression vectors, bacterial expressian vectors and viral expression vectors and are described in Sambrook et al., Molecular Cloning: A laboratory Maunal, Second Edition, Cold Spring Harbor, NY., (1989) A preferred vector is pFLAG-CMV-1 or pcDNA3.

The vector may also include appropriate sequences for selection and/or amplification of expression. For this the vector will comprise one or more phenotypic selectable/amplifiable markers. Such markers are also well knwon to those skilled in the art.

In a further embodiment, the present invention provides host cells comprising a vector of the invention, and capable of expressing a nucleotide sequence of the invention. The host cells can be, for example, a higher eukaryotic cell, such as a mammalian cell or a lower eukaryotic cell, such as a yeast cell or a prokaryotic

cell such as a bacterial cell. Suitable prokaryotic hosts for transformation include E-coli. Suitable eukaryotic hosts include HEK293T cells and HeLa cells.

Cell free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

Routine methods can be employed to purify the protein of the invention from recombinant cell cultures. Such methods are well understood by persons skilled in the art.

The proteins and nucleotide sequences of the present invention are provided in an isolated form. The term "isolated" is intended to convey that the the material is not in its native state. Thus, the naturally-occurring nucleotide sequence or protein present in a living animal is in its native state and is not isolated, but the same nucleotide sequence or protein, separated from some or all of the materials it co-exists with in the natural system, is isolated. Similarly, a protein which has been produced by synthetic means, for example, by recombinant methods is "isolated." Such nucleotide sequence could be part of a vector. Such nucleotide sequence or protein could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The proteins and nucleotide sequences of the present invention are also preferably provided in purified form, and preferably are purified to at least 50% purity, more preferably about 75% purity, most preferably 90% purity or greater, such as 95%, 98% pure.

A further aspect of the present invention is the use of the proteins according to the invention in screening methods. Such methods identify compounds which act as modulators of the interaction between proteins of the invention and their receptor. In general terms, such screens will comprise contacting a protein of the invention, preferably in trimeric form, and its receptor in the presence or absence of the test compound, and measuring the increase or decrease in the level of binding when the test compound is present. The proteins of the invention may be used in high throughput screens, thus enabling large numbers of compounds to be studied. The screening methods of the invention are generally well known to persons skilled in the art. The present invention also includes within its scope

those compounds which are identified by the screening methods of the invention as possessing useful activity.

The present invention further provides compounds which are modulators of the interaction between a protein of the invention and its receptor for use in therapy, for example immunotherapy. The compounds are provided for use in the treatment of, for example, autoimmune disease, inflammation and other diseases associated with the activation of the transcription factor NF-κB, for example, rheumatoid arthritis, in the treatment of cancers, in the treatment of infections, such as septic shock and in the treatment of atherosclerosis. Generally the compounds are antagonists of the receptor to which the proteins of the invention bind.

The present invention further provides the protein of the invention for use in therapy, for example, for use in immunotherapy, particularly during viral infections, or as a vaccine adjuvant.

The invention further provides the use of compounds which have been identified by the screening techniques of the invention, for the manufacture of a medicament for use in treatment or prophylaxis of disorders that are responsive to modulation of the interaction between the protein of the invention and its receptor.

The present invention also provides the use of the protein of the invention in the manufacture of a medicament for use in immunotherapy, for example, during viral infections, or as a vaccine adjuvant.

The present invention additionally provides a method of treatment of a disorder which is responsive to modulation of the interaction between the protein of the invention and its receptor which comprises administering to a patient an effective amount of a compound identifiable by the screening techniques of the invention, or an effective amount of the protein of the invention.

The invention also provides a nucleotide sequence as defined herein, for use in gene therapy, for example, to increase the production of the protein of the

invention in disorders which respond to an increased level of the protein of the sequence of Fig 1 or Fig 2.

Complementary or anti-sense strands of the nucleotide sequences of the invention can also be used in gene therapy. For example, a cDNA sequence or fragments thereof could be used in gene therapy strategies to down regulate expression of the protein of the invention. Antisense technology can be used to control gene expression through triple-helix formation of antisense DNA or RNA, both of which methods are based on binding of a nucleotide sequence to DNA or RNA.

The present invention further provides a method of producing a protein of the invention, which method comprises introducing into an appropriate cell line a vector comprising a polynucleotide as defined herein under conditions suitable for obtaining expression of the protein

Brief Description of the Figures:

Figure 1 shows the amino acid sequence of the soluble human form of the protein of the invention. Receptor binding sites are shown in bold, and potential N-linked glycosylation sites are marked with a dot.

Figure 2 shows the amino acid sequence of the human membrane bound form of the protein of the invention, which comprises within it the soluble form as shown in figure 1. Annotations are as for figure 1. The transmembrane sequence is underlined.

Figure 3 shows the cDNA nucleotide sequence which encodes the amino acid sequence as shown in figure 1, aligned to the corresponding amino acid sequence. Receptor binding regions are boxed, and potential N-linked glycosylation sites are marked with a dot.

Figure 4 shows the cDNA nucleotide sequence which encodes the amino acid sequence as shown in figure 2, aligned to the corresponding amino acid

sequence. Annotations are as for figure 3. The transmembrane sequence is underlined.

Figure 5 shows the amino acid sequence of the soluble mouse form of the protein of the invention. The N-linked glycosylation site is marked with a dot.

Figure 6 shows the amino acid sequence of the mouse membrane bound form of the protein of the invention. Annotations are as for figure 5. The transmembrane region is underlined.

Figure 7 shows the cDNA nucleotide sequence that encodes the amino acid sequence as shown in figure 5, aligned with the corresponding amino acid sequence. Annotations are as for figure 5.

Figure 8 shows the cDNA nucleotide sequence that encodes the amino acid sequence as shown in figure 6, aligned with the corresponding amino acid sequence. Annotations are as for figure 6.

Figure 9 shows an alignment between the mouse and human forms of the full length form of the protein of the invention.

Figure 10 shows Northern Blot analysis of the tissue specific expression of the protein of the invention in normal mouse (A) and human (B) tissues.

Figure 11 shows Northern Blot analysis of the tissue specific expression of the protein of the invention in immune related tissue (A) and human tumour cell lines (B).

Figure 12 shows cell binding data of FLAG-sD7 (as defined in example 3 below). The shaded area indicates binding to the B-cell lymphoma cell line RPMI 8866. In the absence of FLAG-sD7 no binding is seen (dotted line)

Throughout the examples:

the protein having the amino acid sequence as shown in figure 1 will be termed soluble D7 ligand, and the protein having the amino acid sequence as shown in figure 2 will be termed D7 ligand.

Example 1: Use of soluble D7 ligand in a screen to identify compounds that modulate the interaction between the D7 ligand and its receptor.

All incubations are done at room temperature

Costar RIA/EIA high binding plates are coated with goat anti-human IgG (Sigma I3382) at 2μg/ml in PBS overnight. The coating antibody is removed, and the plates are blocked for at least 2 hours in PBS/2% (w/v) BSA. Plates are then washed three times with PBS/0.1% (v/v) Tween20.
100μl receptor-Fc (1μg/ml) in PBS/1% (w/v) BSA/0.1% (v/v) Tween20 is added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.
Biotin-soluble D7 ligand dilutions in PBS/1% BSA/0.1% Tween20 are added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.
Streptavidin alkaline phosphatase (1:1000) (Amersham RPN1234) is added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.
Binding is detected using Life Technologies amplifier solutions (19589-019).

Example 2: A cell based screen to identify compounds that modulate the interaction between the D7 ligand and its receptor

A B cell line known to bind and respond to the D7 ligand is treated with recombinant soluble human D7 ligand (e.g. FLAG-shD7 as exemplified below) for a defined time.
Cells are harvested, and the response assayed (The response may be possibly proliferation, apoptosis or NF-κB activation).
The assay enables determination of whether the addition of compounds inhibits the induction of a response in target cells.

Example 3: Synthesis and Purification of the soluble D7 ligand

Nucleic acid encoding the soluble human D7 ligand (amino acids 133 to 285) was generated by PCR using the cloned full-length open reading frame as a template.

Nucleic acid encoding the soluble human ligand D7 was cloned into vector pFLAG-CMV-1 (Kodak) (containing a CMV promoter, a preprotrypsin leader sequence, an amino-terminal FLAG epitope and a human growth hormone polyA addition sequence) to form construct pFLAG-CMV-1-hsD7.

5×10^6 HEK 293T cells were resuspended in 250µl cytomix (120mM KCl; 0.15mM $CaCl_2$; 10mM K_2HPO_4/KH_2PO_4, pH 7.6; 25mM Hepes, pH 7.6; 2mM EGTA, pH7.6; 5mM $MgCl_2$; 2mM ATP; 5mM glutathione; pH adjusted with KOH) containing 25µg pFLAG-CMV-1-hsD7. Transfection was carried out by using a BioRad gene pulser (960µF, 270V).

Following transfection, cells were left on ice for 10 min, then transferred to a $75cm^2$ tissue culture flask containing 15ml medium (DMEM, 10% FCS, 2mM L-glutamine, penicillin (5µg/ml) and streptomycin (5µg/ml)). Medium containing secreted ligand was harvested after 48h and applied to an affinity chromatography column containing anti-FLAG M2 antibody coupled to agarose (Kodak). This was washed with Tris-buffered saline (pH 7.4) and fractions were eluted in 0.1M citrate buffer (pH 2.5). Fractions were immediately neutralised with 0.2 volumes 1M Tris.HCl (pH 7.6).

Fractions containing a human soluble D7 ligand linked to the FLAG epitope (FLAG-hsD7) were identified by Western blotting using M2 anti-FLAG antibody. These fractions were pooled, and concentrated using a Centricon Plus-20 (NWML 5000) column (Millipore). FLAG-hsD7 ligand was stored at -70°

Example 4: Synthesis and Purification of the D7 ligand

The open reading frame of human D7 ligand is cloned into vector pcDNA3 (containing a CMV promoter and a bovine growth hormone polyA addition signal) to form construct pcDNA3-hD7.

Plasmid pcDNA3-hD7 is transiently transfected by electroporation into HEK 293T cells (protocol as in example 3).

Cells are harvested after 48h, and homogenised using a Dounce homogeniser in three volumes of protein extraction buffer (25mM Hepes pH 7.4, 0.5% Triton-X-100, 1 "complete" protease inhibitor cocktail tablet (Boehringer Mannheim) per 50ml buffer).

The D7 ligand is purified by affinity chromatography using anti-D7 antibody coupled to agarose.

Example 5: Northern Blot analysis of the tissue distribution of the D7 ligand.

cDNA coding for human D7 ligand was excised from pCDNA3-hD7 (see example 4) with the restriction enzymes BamH1 and Xba1. This cDNA fragment was labelled with ^{32}P dCTP using the Amersham ready-prime system according to the manufacturers protocol. A 5µl aliquot of this mixture was mixed with 10 ml Expresshyb solution (Clontech 8015-1) and the resulting mixture was incubated with one of the following clontech blots: Mouse (7762-1), Human-1 (#7760), Human Cancer Cell line (#7757) or Human Immune System II (#7768-1); for 2 hours at 65°C with shaking. The probe solution was then removed and the blot was washed successively with 2X SSC (saline sodium citrate), 0.05% SDS at room temperature for three 20 minute periods. This was followed with one wash with 0.1% SSC, 0.1% SDS at room temperature. The blot was then exposed to Kodak XAR-5 film at –70°C for 48 hours.

The results of the Northern blot analysis show that D7 ligand RNA is expressed in heart, lung, spleen, kidney and skeletal muscle but not brain in both mice (figure 10A) and humans (figure 10B). A blot of immune-related tissues demonstrates strong expression of D7 ligand RNA in spleen, lymph node, thymus, appendix, bone marrow and peripheral blood leukocytes (fig11A) supporting its potential role as a regulator of immune system functions. Analysis of RNA from a range of human tumour cell lines shows expression of D7 ligand RNA in HL-60 promyelocytic leukemia cells but not in a range of other tumour cell lines (Figure 11B). The presence of D7 ligand in a leukemic cell line also

supports the fact that the D7 ligand is involved in immune system regulation and disorders.

Example 6: Detection of cell surface binding of FLAG-sD7

10^6 cells were incubated with 50ng FLAG-hsD7 ligand (see example 3) in binding buffer (PBS/2.5% FCS/0.1% sodium azide) for 10 minutes at room temperature. After washing once in binding buffer, cells were incubated with 1μg anti-FLAG M2 antibody for 10 minutes at room temperature. Cells were washed once in binding buffer, then incubated with 150μg phycoerythrin-conjugated anti-mouse antibody for 10 minutes at room temperature. Following two further washes in binding buffer, flow cytometry was performed using a Coulter XL benchtop flow cytometer and data were collected on 10^4 viable cells.

Results of one such experiment are shown in figure 12. No significant signal was detectable when any of the lines tested were treated with anti-FLAG M2 antibody and R-phycoerythrin-conjugated second antibody only, but after prior treatment with FLAG-sD7, the signal clearly demonstrates that FLAG-hsD7 binds to the B lymphoma cell line RPMI 8866. Experiments with other cell lines have shown that FLAG-hsD7 binds two other B cell lines (RPMI 8226 and Raji) but does not bind to the T cell lines H9 and Jurkat, or the myelomonocytic lineage lines HL-60, U937 or THP-1. These results show that the extracellular domain of human D7 ligand binds to B cells, supporting its potential role in regulation of the immune system.

Claims

1 An isolated protein comprising

i) a polypeptide having the amino acid sequence of figure 1; or

ii) a variant of the polypeptide of (i).

2 A protein according to claim 1 wherein the polypeptide (i) has the amino acid sequence of figure 2.

3 A protein according to claim 1 or 2 which comprises a polypeptide which is at least 65% homologous to the amino acid sequence of Fig. 1.

4 An isolated polynucleotide which encodes a protein as claimed in any one of the preceding claims or a strand which is complementary to said polynucleotide.

5 An isolated polynucleotide according to claim 4 which comprises

i) a polynucleotide portion having the sequence as shown in figure 3; or

(ii) a variant of said portion or a complementary strand thereto.

6 An isolated polynucleotide according to claim 4 or 5 wherein the polynucleotide portion has the sequence as shown in figure 4.

7 A vector comprising a polynucleotide as claimed in claim 4, 5 or 6.

8 A host cell comprising a vector as claimed in claim 7.

9 An antibody specific for a protein as claimed in any one of claims 1 to 3.

10 A method for the identification of a compound which modulates the interaction between the protein as claimed in claims 1, 2 or 3 and its receptor, comprising contacting said protein and receptor in the presence of a test compound and monitoring for modulation of the interaction.

11 A compound identifiable by the method according to claim 10 for use in therapy.

12 Use of a compound identifiable by the method of claim 10 for the manufacture of a medicament for use in immunotherapy.

13 A method of treatment of a disorder which is responsive to modulation of the interaction between the protein of claims 1, 2 or 3 and its receptor which comprises administering to a patient an effective amount of a compound identifiable by a method according to claim 10.

14 The method according to claim 13 wherein the disorder is a disorder of the immune system, or cancer.

15 A protein according to claim 1, 2 or 3 for use in therapy.

16 Use of a protein according to claims 1 to 3 for the manufacture of a medicament for use in immunotherapy or treatment of cancer.

18 A method of treatment of a disorder which is responsive to modulation of the interaction between the protein of claim 1, 2 or 3 and its receptor which comprises administering to a patient an effective amount of a protein according to any of claims 1 to 3.

19 A method of producing a protein as claimed in claim 1, 2 or 3 which method comprises introducing into an appropriate cell line a vector comprising a polynucleotide as claimed in any one of claims 4 to 6 under conditions suitable for obtaining expression of the protein.

Figure 1

133

RAVQGPEET 9

VTQDCLQLIAD**SETPTI**QKGSYTFV**PWLLS** . . 30

FKRGSALEEKENKIL**VKET**GYFFIYGQVLY 50

TDKTYAMGHLIQRKKVHVFGDELSLVTLFR 30

CIQNMPETLPNNSCYSAGIAKLEEGDELQL 30 .

AIPRE**NAQISLDG**DVTFFGALKLL 24 .

285

(153.)

285
153
132

Figure 2

MDDSTEREQSRLTSCLKKREE

MKLKECVSILPRKESPSVRSSKDGKLLAAT

LLLALLSCCLTVVSFYQVAALQGDLASLRA

ELQGHHAEKLPAGAGAPKAGLEETPAVTAG

LKIFEPPAPGEGNSSQNSRNKRAVQGPEET

VTQDCLQLIAD**SETPTI**QKGSYTFV**PWLLS**

FKRGSALEEKENKIL**VKET**GYFFIYGQVLY

TDKTYAMGHLIQRKKVHVFGDELSLVTLFR

CIQNMPETLPNNSCYSAGIAKLEEGDELQL

AIPRE**NAQISLDGD**VTFFGALKLL

Figure 3

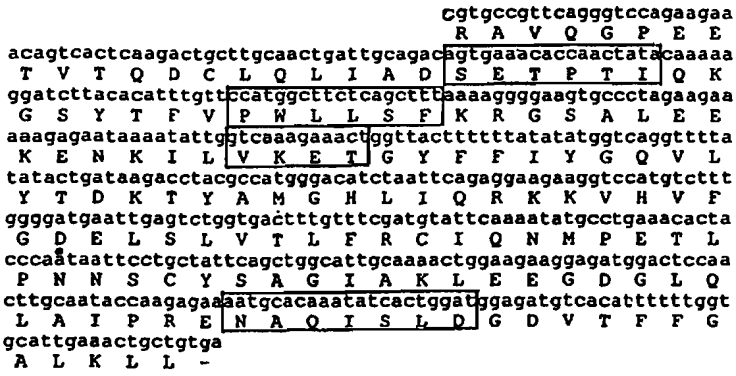

cgtgccgttcagggtccagaagaa
R A V Q G P E E
acagtcactcaagactgcttgcaactgattgcagacagtgaaacaccaactatacaaaaa
T V T Q D C L Q L I A D S E T P T I Q K
ggatcttacacatttgttccatggcttctcagctttaaaaggggaagtgccctagaagaa
G S Y T F V P W L L S F K R G S A L E E
aaagagaataaaatattggtcaaagaaactggttacttttttatatatggtcaggtttta
K E N K I L V K E T G Y F F I Y G Q V L
tatactgataagacctacgccatgggacatctaattcagaggaagaaggtccatgtcttt
Y T D K T Y A M G H L I Q R K K V H V F
ggggatgaattgagtctggtgactttgtttcgatgtattcaaaatatgcctgaaacacta
G D E L S L V T L F R C I Q N M P E T L
cccaataattcctgctattcagctggcattgcaaaactggaagaaggagatggactccaa
P N N S C Y S A G I A K L E E G D G L Q
cttgcaataccaagagaaaatgcacaaatatcactggatggagatgtcacatttttggt
L A I P R E N A Q I S L D G D V T F F G
gcattgaaactgctgtga
A L K L L -

Figure 4

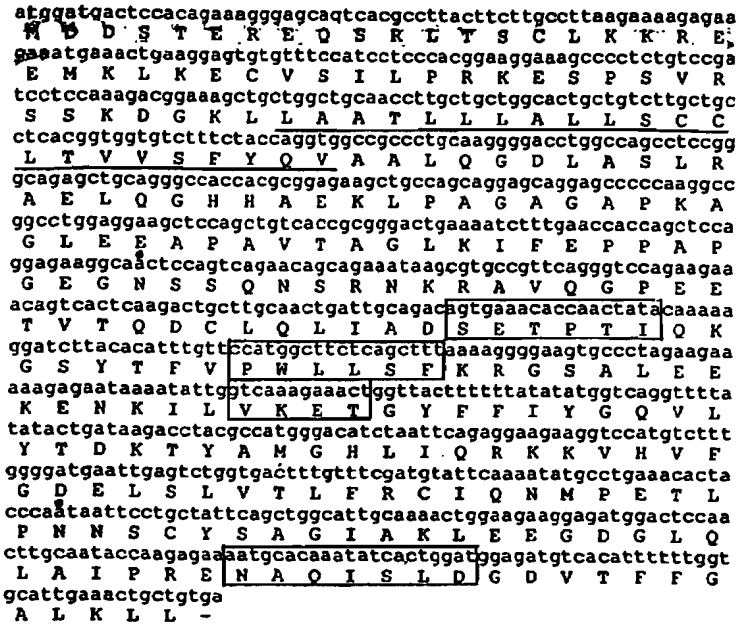

atggatgactccacagaaagggagcagtcacgccttacttcttgccttaagaaaagagaa
M D D S T E R E Q S R L T S C L K K R E
gaaatgaaactgaaggagtgtgtttccatcctcccacggaaggaaagcccctctgtccga
E M K L K E C V S I L P R K E S P S V R
tcctccaaagacggaaagctgctggctgcaaccttgctgctggcactgctgtcttgctgc
S S K D G K L L A A T L L L A L L S C C
ctcacggtggtgtctttctaccaggtggccgccctgcaaggggacctggccagcctccgg
L T V V S F Y Q V A A L Q G D L A S L R
gcagagctgcagggccaccacgcggagaagctgccagcaggagcaggagcccccaaggcc
A E L Q G H H A E K L P A G A G A P K A
ggcctggaggaagctccagctgtcaccgcgggactgaaaatctttgaaccaccagctcca
G L E E A P A V T A G L K I F E P P A P
ggagaaggcaactccagtcagaacagcagaaataagcgtgccgttcagggtccagaagaa
G E G N S S Q N S R N K R A V Q G P E E
acagtcactcaagactgcttgcaactgattgcagacagtgaaacaccaactatacaaaaa
T V T Q D C L Q L I A D S E T P T I Q K
ggatcttacacatttgttccatggcttctcagctttaaaaggggaagtgccctagaagaa
G S Y T F V P W L L S F K R G S A L E E
aaagagaataaaatattggtcaaagaaactggttacttttttatatatggtcaggtttta
K E N K I L V K E T G Y F F I Y G Q V L
tatactgataagacctacgccatgggacatctaattcagaggaagaaggtccatgtcttt
Y T D K T Y A M G H L I Q R K K V H V F
ggggatgaattgagtctggtgactttgtttcgatgtattcaaaatatgcctgaaacacta
G D E L S L V T L F R C I Q N M P E T L
cccaataattcctgctattcagctggcattgcaaaactggaagaaggagatggactccaa
P N N S C Y S A G I A K L E E G D G L Q
cttgcaataccaagagaaaaatgcacaaatatcactggatggagatgtcacatttttggt
L A I P R E N A Q I S L D G D V T F F G
gcattgaaactgctgtga
A L K L L -

Figure 5

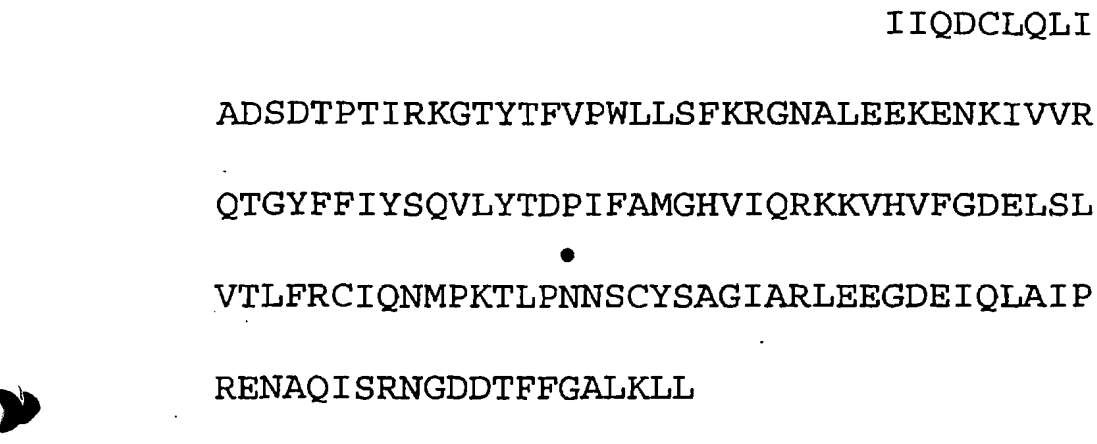

IIQDCLQLI
ADSDTPTIRKGTYTFVPWLLSFKRGNALEEKENKIVVR
QTGYFFIYSQVLYTDPIFAMGHVIQRKKVHVFGDELSL
VTLFRCIQNMPKTLPNNSCYSAGIARLEEGDEIQLAIP
RENAQISRNGDDTFFGALKLL

Figure 6

MDESAKTLPPPCLCFCSEKGED

MKVGYDPITPQKEEGAWFGICRDGRLLAATLLLALLSS

SFTAMSLYQLAALQADLMNLRMELQSYRGSATPAAAGA

PELTAGVKLLTPAAPRPTTPSRGHRNRRAFQGPEETEQ

DVDLSAPPAPCLPGCRHSQHDDNGMNLRNIIQDCLQLI

ADSDTPTIRKGTYTFVPWLLSFKRGNALEEKENKIVVR

QTGYFFIYSQVLYTDPIFAMGHVIQRKKVHVFGDELSL

VTLFRCIQNMPKTLPNNSCYSAGIARLEEGDEIQLAIP

RENAQISRNGDDTFFGALKLL

Figure 7

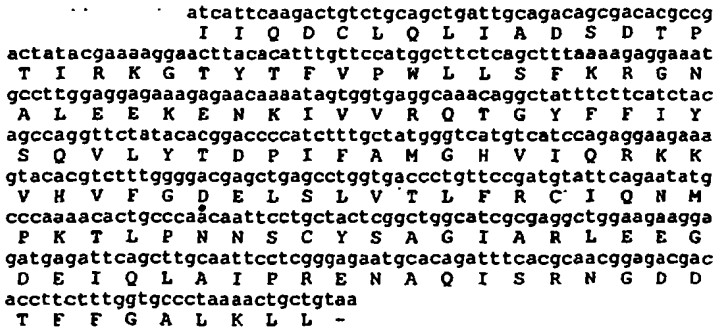

atcattcaagactgtctgcagctgattgcagacagcgacacgccg
I I Q D C L Q L I A D S D T P
actatacgaaaaggaacttacacatttgttccatggcttctcagctttaaaagaggaaat
T I R K G T Y T F V P W L L S F K R G N
gccttggaggagaaagagaacaaaatagtggtgaggcaaacaggctatttcttcatctac
A L E E K E N K I V V R Q T G Y F F I Y
agccaggttctatacacggaccccatctttgctatgggtcatgtcatccagaggaagaaa
S Q V L Y T D P I F A M G H V I Q R K K
gtacacgtctttggggacgagctgagcctggtgaccctgttccgatgtattcagaatatg
V H V F G D E L S L V T L F R C I Q N M
cccaaaacactgcccaacaattcctgctactcggctggcatcgcgaggctggaagaagga
P K T L P N N S C Y S A G I A R L E E G
gatgagattcagcttgcaattcctcgggagaatgcacagatttcacgcaacggagacgac
D E I Q L A I P R E N A Q I S R N G D D
accttctttggtgccctaaaactgctgtaa
T F F G A L K L L -

Figure 8

```
atggatgagtctgcaaagaccctgccaccaccgtgcctctgtttttgctccgagaaagga
 M  D  E  S  A  K  T  L  P  P  P  P  C  L  C  F  C  S  E  K  G
gaagatatgaaagtgggatatgatcccatcactccgcagaaggaggagggtgcctggttt
 E  D  M  K  V  G  Y  D  P  I  T  P  Q  K  E  E  G  A  W  F
gggatctgcagggatggaaggctgctggctgctaccctcctgctggccctgttgtccagc
 G  I  C  R  D  G  R  L  L  A  A  T  L  L  L  A  L  L  S  S
agtttcacagcgatgtccttgtaccagttggctgccttgcaagcagacctgatgaacctg
 S  F  T  A  M  S  L  Y  Q  L  A  A  L  Q  A  D  L  M  N  L
cgcatggagctgcagagctaccgaggttcagcaacaccagccgccgcgggtgctccagag
 R  M  E  L  Q  S  Y  R  G  S  A  T  P  A  A  A  G  A  P  E
ttgaccgctggagtcaaactcctgacgccggcagctcctcgaccccacaactccagccgc
 L  T  A  G  V  K  L  L  T  P  A  A  P  R  P  H  N  S  S  R
ggccacaggaacagacgcgctttccagggaccagaggaaacagaacaagatgtagacctc
 G  H  R  N  R  R  A  F  Q  G  P  E  E  T  E  Q  D  V  D  L
tcagctcctcctgcaccatgcctgcctggatgccgccattctcaacatgatgataatgga
 S  A  P  P  A  P  C  L  P  G  C  R  H  S  Q  H  D  D  N  G
atgaacctcagaaacatcattcaagactgtctgcagctgattgcagacagcgacacgccg
 M  N  L  R  N  I  I  Q  D  C  L  Q  L  I  A  D  S  D  T  P
actatacgaaaaggaacttacacatttgttccatggcttctcagctttaaaagaggaaat
 T  I  R  K  G  T  Y  T  F  V  P  W  L  L  S  F  K  R  G  N
gccttggaggagaaagagaacaaaatagtggtgaggcaaacaggctatttcttcatctac
 A  L  E  E  K  E  N  K  I  V  V  R  Q  T  G  Y  F  F  I  Y
agccaggttctatacacggacccatctttgctatgggtcatgtcatccagaggaagaaa
 S  Q  V  L  Y  T  D  P  I  F  A  M  G  H  V  I  Q  R  K  K
gtacacgtctttggggacgagctgagcctggtgaccctgttccgatgtattcagaatatg
 V  H  V  F  G  D  E  L  S  L  V  T  L  F  R  C  I  Q  N  M
cccaaaacactgcccaacaattcctgctactcggctggcatcgcgaggctggaagaagga
 P  K  T  L  P  N  N  S  C  Y  S  A  G  I  A  R  L  E  E  G
gatgagattcagcttgcaattcctcgggagaatgcacagatttcacgcaacggagacgac
 D  E  I  Q  L  A  I  P  R  E  N  A  Q  I  S  R  N  G  D  D
accttctttggtgccctaaaactgctgtaa
 T  F  F  G  A  L  K  L  L  -
```

Figure 9

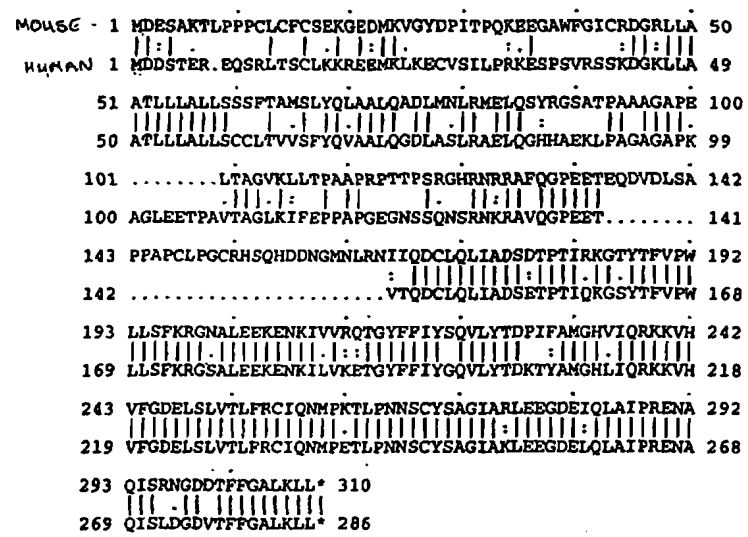

MOUSE - 1 MDESAKTLPPPCLCFCSEKGEDMKVGYDPITPQKEEGAWFGICRDGRLLA 50
HUMAN 1 MDDSTER.EQSRLTSCLKKREEMKLKECVSILPRKESPSVRSSKDGKLLA 49
51 ATLLLALLSSSFTAMSLYQLAALQADLMNLRMELQSYRGSATPAAAGAPE 100
50 ATLLLALLSCCLTVVSFYQVAALQGDLASLRAELQGHHAEKLPAGAGAPK 99
101 .....LTAGVKLLTPAAPRPTTPSRGHRNRRAFQGPEETEQDVDLSA 142
100 AGLEETPAVTAGLKIFEPPAPGEGNSSQNSRNKRAVQGPEET..... 141
143 PPAPCLPGCRHSQHDDNGMNLRNIIQDCLQLIADSDTPTIRKGTYTFVPW 192
142 .....VTQDCLQLIADSETPTIQKGSYTFVPW 168
193 LLSFKRGNALEEKENKIVVRQTGYFFIYSQVLYTDPIFAMGHVIQRKKVH 242
169 LLSFKRGSALEEKENKILVKETGYFFIYGQVLYTDKTYAMGHLIQRKKVH 218
243 VFGDELSLVTLFRCIQNMPKTLPNNSCYSAGIARLEEGDEIQLAIPRENA 292
219 VFGDELSLVTLFRCIQNMPETLPNNSCYSAGIAKLEEGDELQLAIPRENA 268
293 QISRNGDDTFFGALKLL* 310
269 QISLDGDVTFFGALKLL* 286

Figure 10

A

1 = heart
2 = brain
3 = spleen
4 = lung
5 = liver
6 = skeletal muscle
7 = kidney
8 = testis

B

1 = heart
2 = brain
3 = placenta
4 = lung
5 = liver
6 = skeletal muscle
7 = kidney
8 = pancreas

Figure 11

A

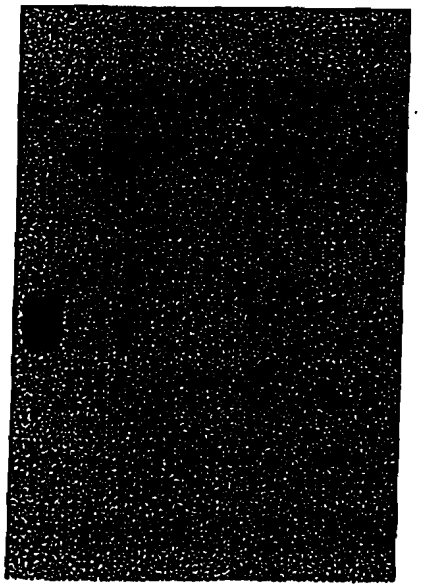
1 2 3 4 5 6 7

2.6kB

1 = spleen
2 = lymph node
3 = thymus
4 = appendix
5 = PBL
6 = bone marrow
7 = foetal liver

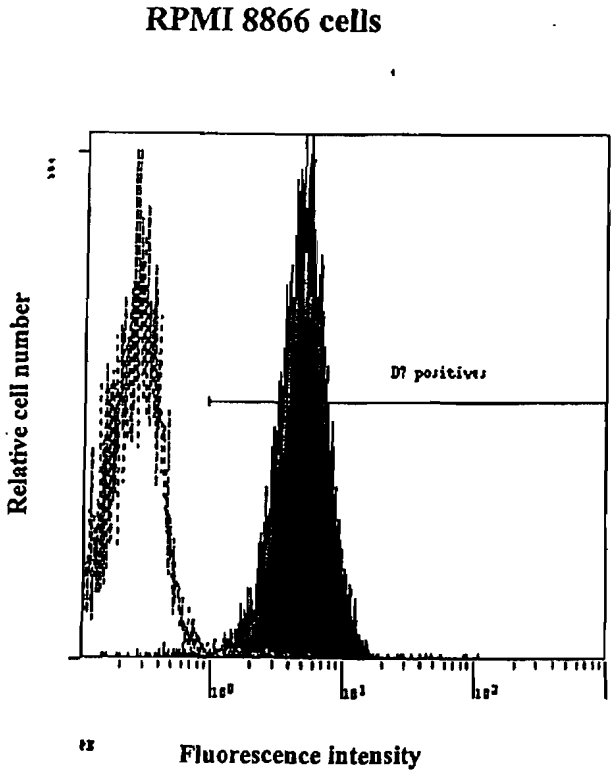
B

1 2 3 4 5 6 7 8



1 = HL60
2 = HeLa S3
3 = K562
4 = Molt-4
5 = Raji
6 = SW480
7 = A549
8 = G361

Figure 12


RPMI 8866 cells
Relative cell number
D7 positives
Fluorescence intensity